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25 October 2001

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

(54) Title: THERMOSTABLE ENZYME PROMOTING THE FIDELITY OF THERMOSTABLE DNA POLYMERASES- FOR IMPROVEMENT OF NUCLEIC ACID SYNTHESIS AND AMPLIFICATION *IN VITRO*

Error rates of different DNA polymerases in PCR

Polymerase	Template conc. (ng)	yield (ng)	DNA duplications d	blue colonies lacI⁺	white colonies lac⁻	total number of colonies	% lac⁻	error rate (f_{lacI})
Taq Ch.	10	11650	10.2	130	2261	2391	5.4	1.57×10^{-4}
HiFi Ch.	10	11550	10.2	40	5458	5498	0.72	2.06×10^{-5}
Pwo	10	9875	9.9	17	5891	5908	0.29	8.32×10^{-6}
Taq/Exo 1	10	11550	10.2	94	4291	4385	2.14	6.10×10^{-5}
Taq/Exo 2	10	11125	10.1	146	7644	7790	1.87	5.36×10^{-5}
Taq/Exo 3	10	8500	9.7	133	8188	8321	1.6	4.74×10^{-5}
Taq/Exo 4	10	1292	7	79	7236	7315	1.08	4.44×10^{-5}
Taq/Exo 5	10	236	4.6	25	2674	2724	0.92	1.16×10^{-5} *

* Due to the unfavorable ratio of Taq:Exo the product yield was low. This results in an apparently low amplification efficiency d, which is an important parameter in the formula used for the calculation of the error rate.

(57) Abstract: A purified thermostable enzyme is derived from the thermophilic archaebacterium *Archaeoglobus fulgidus*. The enzyme can be native or recombinant, is stable under PCR conditions and exhibits double strand specific exonuclease activity. It is a 3'-5' exonuclease and cleaves to produce 5'-mononucleotides. Thermostable exonucleases are useful in many recombinant DNA techniques, in combination with a thermostable DNA polymerase like *Taq* especially for nucleic acid amplification by the polymerase chain reaction (PCR).

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A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 C12N15/55 C12N15/54 C12N15/10 C12N9/22 C12N9/12
C12Q1/68 C12P19/34

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 C12N C12Q C12P

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)
WPI Data, PAJ, CAB Data, STRAND, EPO-Internal

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	H.P. KLENK ET AL.: "Archaeoglobus fulgidus section 43 of 172 of the complete genome" EMBL SEQUENCE DATABASE, 1 December 1997 (1997-12-01), XP002139308 Hinxton, UK cited in the application	1-3
Y	Accession no. AE001064, TrEMBLrel 029675, 01-01-1998; EXODEOXYRIBONUCLEASE III (XTHA);	17,18
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[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

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"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
10 May 2001	17. 05. 01

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Hornig, H

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C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	"Archaeoglobus fulgidus genome" THE INSTITUTE FOR GENOME RESEARCH; TIGR DATABASE, 15 May 1999 (1999-05-15), XP002139309 US cited in the application	1-3
Y	Locus AF0580, exodeoxyribonuclease III (xthA);	17,18
X	KLENK H-P ET AL: "The complete genome sequence of the hyperthermophilic, sulphate-reducing archaeon Archaeoglobus fulgidus" NATURE,GB,MACMILLAN JOURNALS LTD. LONDON, vol. 390, 27 November 1997 (1997-11-27), pages 364-370, XP002091622 ISSN: 0028-0836 cited in the application	1-3
Y	Accession no. AE001064; SPTREMBL: 029675, exodeoxyribonuclease III (xthA);	17,18
X	WO 99 13060 A (ENZYCO INC) 18 March 1999 (1999-03-18) cited in the application the whole document	1,3
X	DE 198 10 879 A (BIOTECHNOLOG FORSCHUNG GMBH ;ROCHE DIAGNOSTICS GMBH (DE)) 16 September 1999 (1999-09-16) page 3, line 55 - line 59 page 4, line 40 - line 56	1,4
X	EP 0 870 832 A (TAKARA SHUZO CO) 14 October 1998 (1998-10-14) the whole document	17
X	EP 0 744 470 A (JOHNSON & JOHNSON CLIN DIAG) 27 November 1996 (1996-11-27) the whole document	17
X	WO 94 26766 A (BARNES WAYNE M) 24 November 1994 (1994-11-24) the whole document	17
X	EP 0 669 401 A (HOFFMANN LA ROCHE) 30 August 1995 (1995-08-30) the whole document	17

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INTERNATIONAL SEARCH REPORT

International application No
PCT/EP 00/09423

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	ZHU Y S ET AL: "THE USE OF EXONUCLEASE III FOR POLYMERASE CHAIN REACTION STERILIZATION" NUCLEIC ACIDS RESEARCH,GB,OXFORD UNIVERSITY PRESS, SURREY, vol. 19, no. 9, 28 January 1991 (1991-01-28), page 2511 XP000199115 ISSN: 0305-1048 page 2511, left-hand column, line 21 - line 26 page 2511, right-hand column, line 3 - line 5 ---	17,18
A	KALUZ S ET AL: "DIRECTIONAL CLONING OF PCR PRODUCTS USING EXONUCLEASE III" NUCLEIC ACIDS RESEARCH,GB,OXFORD UNIVERSITY PRESS, SURREY, vol. 20, no. 16, 1 January 1992 (1992-01-01), pages 4369-4370, XP002072726 ISSN: 0305-1048 the whole document ---	17,18
A	WO 98 45452 A (UNIV ROCKEFELLER) 15 October 1998 (1998-10-15) cited in the application the whole document ---	
A	WO 94 23066 A (US BIOCHEMICAL CORP) 13 October 1994 (1994-10-13) the whole document ---	
A	BOOTH P M ET AL: "ASSEMBLY AND CLONING OF CODING SEQUENCES FOR NEUROTROPHIC FACTORS DIRECTLY FROM GENOMIC DNA USING POLYMERASE CHAIN REACTION AND URACIL DNA GLYCOSYLASE" GENE,NL,ELSEVIER BIOMEDICAL PRESS. AMSTERDAM, vol. 146, no. 2, 1 January 1994 (1994-01-01), pages 303-308, XP002071998 ISSN: 0378-1119 the whole document ---	

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INTERNATIONAL SEARCH REPORT

Internatio[illegible]pplication No
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C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	SMITH C ET AL: "GENERATION OF COHESIVE ENDS ON PCR PRODUCTS BY UDG-MEDIATED EXCISION OF FU, AND APPLICATION FOR CLONING INTO RESTRICTION DIGEST-LINEARIZED VECTORS" PCR METHODS & APPLICATIONS,US,COLD SPRING HARBOR LABORATORY PRESS, vol. 2, no. 4, 1 May 1993 (1993-05-01), pages 328-332, XP002071999 ISSN: 1054-9803 the whole document	
A	AYOUB RASHTCHIAN ET AL: "URACIL DNA GLYCOSYLASE-MEDIATED CLONING OF POLYMERASE CHAIN REACTION-AMPLIFIED DNA: APPLICATION TO GENOMIC AND CDNA CLONING" ANALYTICAL BIOCHEMISTRY,US,ACADEMIC PRESS, SAN DIEGO, CA, vol. 206, no. 1, 1 October 1992 (1992-10-01), pages 91-97, XP000311343 ISSN: 0003-2697 the whole document	
P,A	FROMENTY ET AL: "Escherichia coli exonuclease III enhances long PCR amplification of damaged DNA templates" NUCLEIC ACIDS RESEARCH,OXFORD UNIVERSITY PRESS, SURREY,GB, vol. 28, no. 11, June 2000 (2000-06), page e50 XP002151230 ISSN: 0305-1048 the whole document	
E	WO 00 68411 A (LIFE TECHNOLOGIES INC) 16 November 2000 (2000-11-16) abstract; claims 1-52 page 4, line 25 -page 7, line 18	1,4
E,L	EP 1 088 891 A (ROCHE DIAGNOSTICS GMBH) 4 April 2001 (2001-04-04) L: Priority	1-3,5-16

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INTERNATIONAL SEARCH REPORT

International application No.
PCT/EP 00/09423

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

see additional sheet

1. ☒ As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest.

☒ No protest accompanied the payment of additional search fees.

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

This International Searching Authority found multiple (groups of) inventions in this international application, as follows:

1. Claims: (1-3,5-16)-complete, 18-partially

Thermostable enzyme exhibiting 3'-exonuclease-activity but essentially no DNA polymerase activity whereas this enzyme enhances fidelity of an amplification process when added to a second enzyme exhibiting polymerase activity; said enzyme obtainable from Archaeglobus fulgidus, whereas this enzyme is able to cooperate as proofreading enzyme with a second enzyme exhibiting polymerase activity; Composition comprising a first thermostable enzyme exhibiting 3'-exonuclease-activity but essentially no DNA polymerase activity and a second enzyme exhibiting DNA polymerase activity whereas the fidelity of an amplification process is enhynced by the use of the composition to the use of the single second enzyme; a method of preparing or amplifying DNA using said composition; said method whereas the mixture of a -first thermostable enzyme exhibiting 3'-exonuclease-activity but essentially no DNA polymerase activity and -a second enzyme exhibiting DNA polymerase activity produces PCR products with lower error rates compared to PCR products produced by the second enzyme exhibiting DNA polymerase activity in absence of the first thermostable enzyme exhibiting 3'-exonuclease-activity but essentially no DNA polymerase activity; said method whereas PCR products with blunt ends are obtained; a method for amplifying DNA using said thermostable enzyme exhibiting 3'-exonuclease-activity which enzyme is not or only to a negligible extend active on linear single stranded DNA;

2. Claims: 4-complete, 18-partially

Thermostable enzyme exhibiting 3'-exonuclease-activity but exhibits reduced DNA polymerase activity; a method for amplifying DNA using said thermostable enzyme exhibiting 3'-exonuclease-activity which enzyme is not or only to a negligible extend active on linear single stranded DNA;

3. Claims: 17-complete, 18-partially

A method for amplifying DNA using a thermostable enzyme exhibiting 3'-exonuclease-activity which enzyme is not or only to a negligible extend active on linear single stranded DNA;

INTERNATIONAL SEARCH REPORT

International Application No
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Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9913060 A	18-03-1999	AU 9229698 A	29-03-1999
		EP 1012248 A	28-06-2000
DE 19810879 A	16-09-1999	WO 9947649 A	23-09-1999
EP 0870832 A	14-10-1998	AU 717156 B	16-03-2000
		AU 1209897 A	28-07-1997
		CA 2241170 A	10-07-1997
		CN 1209170 A	24-02-1999
		WO 9724444 A	10-07-1997
EP 0744470 A	27-11-1996	AU 5233996 A	05-12-1996
		CA 2176193 A	23-11-1996
		CN 1140762 A	22-01-1997
		JP 9107997 A	28-04-1997
		NO 962062 A	25-11-1996
WO 9426766 A	24-11-1994	US 5436149 A	25-07-1995
		AT 181573 T	15-07-1999
		AU 671204 B	15-08-1996
		AU 6246494 A	12-12-1994
		CA 2156176 A	24-11-1994
		DE 69419248 D	29-07-1999
		DE 69419248 T	27-01-2000
		DK 693078 T	24-01-2000
		EP 0693078 A	24-01-1996
		ES 2136730 T	01-12-1999
		JP 11239492 A	07-09-1999
		JP 2885324 B	19-04-1999
		JP 11501801 T	16-02-1999
		NZ 262663 A	22-09-1997
EP 0669401 A	30-08-1995	US 5512462 A	30-04-1996
		CA 2143229 A	26-08-1995
		JP 8038198 A	13-02-1996
WO 9845452 A	15-10-1998	AU 7683998 A	30-10-1998
		EP 0983365 A	08-03-2000
WO 9423066 A	13-10-1994	AU 6418294 A	24-10-1994
WO 0068411 A	16-11-2000	AU 5008900 A	21-11-2000
EP 1088891 A	04-04-2001	WO 0123583 A	05-04-2001